

GENE EXPRESSION AND POLYMORPHISMS ASSOCIATED WITH LUNG CANCER

GOVERNMENT RIGHTS

5 [001] This invention was supported, at least in part, by NIH grants R01 CA58554, R01 CA78797, and R01 GM62694. The Federal Government has certain rights in this invention.

PRIORITY CLAIM

[002] This application claims priority to US Provisional Patent Application 60/514,673, filed October 27, 2003, which is incorporated herein by reference, in its entirety.

10

BACKGROUND

[003] Lung cancer is the leading cause of mortality from cancer in both men and women in developed countries. There is evidence that although incidence is almost always associated with environmental factors such as smoking or occupational exposure to carcinogens, susceptibility has a genetic component, with early onset lung cancer following Mendelian inheritance. Moreover, susceptibility is largely intrinsic to the lung itself, as shown by classical experiments involving lung explants from sensitive and resistant mice.

15 [004] Accordingly, it is desirable to have methods to provide additional information about the genetic profile of an individual. This information is useful in the context of cancer, including lung cancer, to identify individuals who are at risk for developing the disease so as to provide preventive care or prophylaxis. It is also useful for determining methods of treatment that are optimized for an individual's particular cancer profile.

20

SUMMARY OF THE INVENTION

[005] This invention relates to diagnosis and treatment of cancer. In particular, it relates to cancers that involve the expression of the Lung Adenoma Susceptibility-1 Gene (Las1) gene and/or its product, the Las1 protein. Las1 is believed to be an inhibitor of cellular proliferation by influencing cell cycle. The Las1 is reported herein by Applicants as a gene

25

that is associated with the pulmonary adenoma susceptibility 1 (Pas1) chromosomal locus that has been described in species such as mice and humans.

[006] Human groups exist that have an above average probability of being diagnosed with and/or of developing particular cancers (i.e., high-risk groups) and are appropriate candidates
5 for evaluation of Las1 expression, and/or therapeutic or prophylactic use of Las1. Such human groups may be at high risk because of exposure to particular environmental materials or circumstances (e.g., smoking or exposure to tobacco smoke, occupational exposure to carcinogens such as urethane and other agents), because of familial susceptibility to certain cancers (e.g., genetic inheritance of genes causing increased susceptibility), as a result of the
10 presence of mutant forms of Las1 or decreased levels of expression of Kirsten rat sarcoma oncogene 2 ("Kras2"), for other reasons, or for combinations of causes and reasons.

[007] In some embodiments, the invention provides methods for characterizing the etiology of a cancer in an individual by testing at least one cancer cell from the individual for at least one of a reduction in the level of expression of Las1 as compared to normal cells, and one or
15 more mutations in the at least one cancer cell's genomic Las1 gene. The levels of Las1 expression in normal cells may be determined from non-cancer cells in the individual, or based on standard levels of Las1 expression in normal cells of other individuals.

[008] According to one embodiment, the at least one cancer cell is tested for the presence of a mutation at codon 60 of the Las1 gene which encodes a mutant Las1 protein.

20 [009] According to other embodiments, the at least one cancer cell is tested for the level of Las1 gene expression. The level of Las1 expression may be tested by measuring mRNA transcribed from the Las1 gene. The level of Las1 expression may be tested by measuring the amount of Las1 protein in the cell. The level of Las1 expression may be tested using an antibody to Las1 protein.

[010] According to one embodiment, the presence of a mutation at codon 60 of the Las1 gene is tested by analyzing the coding sequence of the Las1 gene.

[011] According to one embodiment, the presence of a mutation at codon 60 of the Las1 gene is tested by using an antibody that detects the mutant Las1 protein.

5 [012] According to some embodiments, the methods also comprise testing the at least one cancer cell from the individual for at least one of a reduction in the level of expression, or one or more mutations of one or more of the genes in the Pas-1 locus, which include Kras2, Lrmp, Bcat1, AK016641 and AK015530. In one embodiment, the methods comprise testing the at least one cancer cell from the individual for at least one of a reduction in the level of
10 expression of Kras2 as compared to non-cancer cells from the individual, and one or more mutations in the at least one cancer cell's genomic Kras2 gene. The level of Pas1 gene expression may be tested by measuring mRNA transcribed from one or more of the Pas1 genes. The level of Pas1 expression may be tested by measuring the amount of one or more of the Pas1 gene products in the cell. The level of expression of one or more of the Pas1 gene
15 products may be tested using an antibody to one or more of such gene products. The levels of expression one or more of the Pas1 genes in normal cells may be determined from non-cancer cells in the individual, or based on standard levels of expression of one or more of the Pas1 genes in normal cells of other individuals.

[013] In some embodiments, the invention provides methods for identifying an individual
20 who is at risk of developing cancer by testing at least one cell from the individual for at least one of a reduction in the level of expression of Las1 as compared to normal cells, and one or more mutations in the at least one cell's genomic Las1 gene.

[014] According to one embodiment, the at least one cell is tested for the presence of a mutation at codon 60 of the Las1 gene which encodes a mutant Las1 protein.

[015] According to other embodiments, the at least one cell is tested for the level of Las1 gene expression. The level of Las 1 expression may be tested by measuring mRNA transcribed from the Las1 gene. The level of Las 1 expression may be tested by measuring the amount of Las1 protein in the cell. The level of Las1 expression may be tested using an antibody to Las1 protein.

[016] According to one embodiment, the presence of a mutation at codon 60 of the Las1 gene is tested by analyzing the coding sequence of the Las1 gene.

[017] According to one embodiment, the presence of a mutation at codon 60 of the Las1 gene is tested by using an antibody that detects the mutant Las1 protein.

[018] According to some embodiments, the methods also comprise testing the at least one cell from the individual for at least one of a reduction in the level of expression, or one or more mutations of one or more of the genes in the Pas-1 locus, which include Kras2, Lrmp, Bcat1, AK016641 and AK015530. In one embodiment, the methods comprise testing the at least one cell from the individual for at least one of a reduction in the level of expression of Kras2 as compared to normal cells, and one or more mutations in the at least one cell's genomic Kras2 gene. The level of Pas1 gene expression may be tested by measuring mRNA transcribed from one or more of the Pas1 genes. The level of Pas1 expression may be tested by measuring the amount of one or more of the Pas1 gene products in the cell. The level of expression of one or more of the Pas1 gene products may be tested using an antibody to one or more of such gene products. The levels of expression one or more of the Pas1 genes in normal cells may be determined from other cells in the individual, or based on standard levels of expression of one or more of the Pas1 genes in normal cells of other individuals.

[019] In some embodiments the invention also provides methods for treating an individual identified as having a mutant Las1 gene or reduced expression of Las1 protein by administering to the individual an agent that restores Las1 protein function. The individual

may have an adenocarcinoma, such as an adenocarcinoma of the lung. According to some embodiments, the agent is a Las1 protein. The Las1 protein may be administered in a fashion such that it is specifically targeted to cancer tissue in the individual. According to other embodiments, the agent is a polynucleotide encoding a Las1 protein, wherein the polynucleotide is in operable connection with a promoter that directs its expression. In some embodiments, the treatment is prophylactic.

[020] The genes at the Pas-1 locus, include the Las1, Lrmp, Bcat1, AK016641 and AK015530, and Kras2 genes or ORFs (Figs. 10-19). Sequences for mRNAs encoding Lrmp, Ak015530, and Ak016641 can be found with the National Center for Biotechnology Information GenBank (GI: 6678713, 12853911 and 12855487, respectively). The sequence for Bcat1 can be found in Benvenisty, N. et. al. (1992) Genes Dev., 6, 2513-2523. The sequence for the Kras2 ORF is found in application for US patent, 20030133910. The sequences for Las1 reported in this paper have been deposited in the GenBank database (accession nos. AY423542 for mouse and AY423543 for human. The sequences for these molecules are hereby incorporated by reference, in their entirety.

[021] In some embodiments this invention relates to methods for the treatment of lung cancer, therapeutically to prevent or decrease the proliferation of cancer cells, or prophylactically to prevent formation of lung cancer. Such methods comprise increasing levels of non-mutant Las1 protein, or a functional fragment thereof, in such cells.

[022] In one embodiment, the invention provides a method for therapeutic or prophylactic treatment of a cancer in an individual by administering to the individual one or more agents comprising Las1 protein, or a functional fragment thereof, that inhibits proliferation of cancer cells. A Las1 protein, or a functional fragment thereof, may be administered in a fashion such that it is specifically targeted to cancer tissue. According to this embodiment,

introduction or transfer of a Las1 protein, or a functional fragment thereof, is achieved by any of a variety of methods known in the art to introduce proteins to or into cells.

[023] In another embodiment, the invention provides a method for therapeutic or prophylactic treatment of a cancer in an individual by administering to the individual one or more agents comprising a polynucleotide encoding a Las1 protein, or a functional fragment thereof, wherein the polynucleotide is in operable connection with a promoter that directs its expression.

[024] In one embodiment, the polynucleotide is administered in a fashion such that it is specifically targeted to cancer tissue, and is in an amount sufficient to achieve expression of Las1 protein, or a functional fragment thereof, that inhibits proliferation of cancer cells. According to this embodiment, introduction or transfer of a polynucleotide, such as a DNA molecule or molecules, specifically a DNA molecule encoding one or more Las1 protein, or a functional fragment thereof, into a cell is achieved by any of a variety of methods known in the art to introduce polynucleotides into cells.

[025] In yet another embodiment, the invention provides a method for treating an individual identified as having a disease associated with reduced expression or mutation of Las1 gene by administering to the individual at least one compound that restores the individual's Las1 proteins ability to influence cell cycle and inhibit cancer cell proliferation.

[026] The compositions that are used according to the methods of this invention may be administered prior to, concurrent with, or after administration of other cancer therapeutic or prophylactic treatments. In some embodiments, the agent or agents are administered to an individual identified as having a cancer associated with mutant Las1 genes or reduced expression of Las1. In other embodiments, the agent or agents are administered to an individual having an adenocarcinoma, in particular, an adenocarcinoma of the lung.

[027] Additional features and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The features and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[028] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[029] The accompanying figures, which are incorporated in and constitute a part of this specification, and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[030] Fig. 1 shows the amino acid sequence for Las1 protein from human.

[031] Fig. 2 shows the nucleotide sequence which encodes the human Las1 protein.

[032] Fig. 3. shows the amino acid sequence for the mouse Las1 protein.

[033] Fig. 4 shows the nucleotide sequence which encodes the mouse Las1 protein.

[034] Fig. 5. Characterizations of the Pas1 locus. (A). Substitution mapping of Pas1 QTL for mouse lung tumor susceptibility with the use of a set of congenic strains. The open boxes represent a chromosome fragment from the donor strain (A/J), and the solid boxes represent a chromosome fragment from the recipient strains (C57BL/6J). Eight microsatellite markers were used to allelotype the 26.1 cM region containing the Pas1 locus. AB is a congenic strain in which the entire chromosomal region between markers D6MIT54 and D6MIT373 has been substituted into the recipient C57BL6J strain from the donor A/J strain. Congenic substrains 1 through 8 carry various donor (A/J) fragments as indicated. BB is the control congenic strain in which no substitution was found in the entire region. (B) Expression of

Pas1 candidate genes in mouse lungs. Total RNAs were isolated from A/J and C57BL/6J normal lung tissues. Expression levels of five candidate genes were tested using RT-PCR and Northern blot analysis. For each autoradiograph, Upper panel, individual candidate genes; lower panel, b-actin control. For each candidate gene, the left panel, expression level in C57BL6J lungs; the right panel, expression level in A/J lungs. Note: Bcat1, Lrmp, AK015530, AK016641 are RT-PCR. Kras2 is Northern blot analysis. (C) Functional polymorphisms of Pas1 candidate genes. 1. Sequence analysis of the Las1 gene revealed a functional polymorphism at codon 60 between lung tumor susceptible/intermediate (A/J, SWR/J, BALB/cJ, 129/J, CBA/J, and SM/J) (top) and resistant strains (C57BL/6J, DBA/2J, SJL/J, C3H/HeJ, AKR/J, and Mus. Spretus) (bottom). An amino acid alignment of the codon 52-72 of Las1 is shown, with the asparagine to serine alteration at codon 60 (boxed). 2. Sequence variations of Ak016641 between A/J and C57BL/6J. AK016641 contains 2 functional polymorphisms at codon 218 (Arg to His), codon 258 (Gly to Glu) and an alternative splicing transcript without exon 5 only found in A/J strain. 3. AK015530 had a polymorphism at codon 28 resulting a change of Asp to Gly. 4. Lrmp contains 5 functional polymorphisms including codon 31 (Asp to Gly), codon 56 (Gly to Asp), codon 58 (Phe to Leu), codon 438 (Arg to Gly), and codon 537 (Pro to Leu).

[035] Fig. 6. Cluster alignment of mouse rat human and *Ciona intestinalis* Las1 protein sequences. The sequences of mouse, rat, human, and *Ciona intestinalis* Las1 are presented.

Identical residues are shaded in black. Residues identical in at least two species are shaded in black. In mouse protein, the codon 60 ("x") encodes an Asparagine (AAT) in A/J mice and a Serine (AGT) in C57BL/6J mice. The human protein sequence (67% identities and 81% positives) is based on predicted human Las1 cDNA sequence. Searching NCBI protein database using mouse protein sequence revealed a rat homologous protein, encoded by NCBI predicted gene LOC297720 (84% identities and 92% positives). The mouse Las1 protein is

also homologous to a *Ciona intestinalis* protein axonemal p83.9 (GI: 20086393, 33% identities and 52% positives).

[036] Fig. 7. Characterization of the Las1 gene as a candidate Pas1 gene. **(A)** Colony formation assay. Inhibition of colony formation by transfected Las1 in LM1 cell line (a) and MC14 cell line (b). LM1 and MC14 cells of the same passage were transfected under identical conditions with 4 μ g of purified plasmid DNA of Las1-A/J-pcDNA3.1, Las1-C57BL/6J-pcDNA3.1, or pcDNA3.1 vector alone. 1.5 x 10⁶ cells were seeded into each of the four (10-cm) dishes, and incubated in proper medium plus G418 (50 μ g/ml) for 14 days. The cells were then fixed and stained and colonies with more than 1 mm diameter were counted and the proportions against empty vector (\pm SE) were plotted. Asterisks indicate $P < 0.05$ compared to vector-transfected cells. **(B)** Athymic mouse tumorigenicity assays of Las1 transfected tumor cells. (a) RT-PCR of unique sequences carried by the transfected *Las1* clones. A/J: LM1 cells transfected with Las1-A/J-pcDNA3.1 clone. C57BL/6J: LM1 cells transfected with Las1-C57BL/6J-pcDNA3.1 clone. (b) Measurement of the tumor size. (c), (d), Inhibition of nude mouse tumor development (left side, Las1-A/J-pcDNA3.1 transfected cells; right side, Las1-C57BL/6J-pcDNA3.1 transfected cells). **(C)** Expression of Las1 in mouse tissues and mouse cell lines. (a) Northern blot analysis was used to determine the expression of Las1 in A/J and C57BL/6J lungs. (b), (c), Total RNA was prepared from different mouse tissues and cell lines. RT-PCR analysis was used to determine the expression of Las1 in mouse multiple organ (b) and cell lines (c). β -actin was used as an internal control. **(D)** Subcellular localization of Las1. Myc-tagged Las1 plasmids were transfected into NIH/3T3 and COS7 cells and visualized by immunofluorescent staining using rhodamine (red). Nuclei were stained with DAPI (blue). A/J, Las1-A/J-pcDNA3.1 transfected cells; C57BL/6J, Las1-C57BL/6J-pcDNA3.1 transfected cells, vector, pcDNA3.1 vector transfected cell, control, untransfected negative control cells.

[037] **Fig. 8.** Kras2 allelic effects on chemically induced lung tumorigenesis. (A) Allelic effects on tumor multiplicity. In A/J (open bars) or C57BL/6J (solid bars) hybrid mouse groups, the remaining A/J or C57BL/6J Kras2 allele in Kras2+/- mice has no significant differential effect on lung tumor multiplicity 3.82- (A/J Kras2+/- mice/A/J Kras2+/+ mice) vs 4.88- (C57BL/6J Kras2+/- mice/C57BL/6J Kras2+/+ mice) fold (urethane treated group); 4.76- vs 5.10-fold (MNU treated group) between the two Kras2 alleles. (B) Allelic effects on tumor volume. In A/J or C57BL/6J hybrid groups, the remaining A/J or C57BL/6J Kras2 allele in Kras2+/- mice have a significant differential effect on lung tumor progression 31.73- (A/J Kras2+/- mice/A/J Kras2+/+ mice) vs 8.04- (C57BL/6J Kras2+/- mice/C57BL/6J Kras2+/+ mice) fold (urethane treated group); 56.85- vs 14.47- fold (* p<0.001) between the two Kras2 alleles. (C) Activated Kras2 protein in mouse lung tumors from (A/J~~JK~~-ras+/-) F1, and (C57BL/6J~~JK~~-ras+/-) F1 mice. K-Ras2 activity in large and small tumors from Kras2+/+ and Kras2+/- mice was presented that corresponds to A/J and C57BL/6J alleles. b, and s, correspond to large (> 4 mm in diameter) and small tumors (< 4 mm in diameter), respectively. Note: half the amount of lysate was used in the pull-downs from A/J tumors (lanes 1-4) compared to C57BL/6J tumors (lanes 5-8).

[038] **Fig. 9** shows all genes found in the narrowed Pas1 QTL region encompassed by markers D60SU6 and D60SU12.

[039] **Fig. 10** shows the nucleotide sequence for human LRMP.

[040] **Fig. 11** shows the nucleotide sequence for mouse LRMP.

[041] **Fig. 12** shows the human BCAT1 cDNA sequence.

[042] **Fig. 13** shows the mouse BCAT1 cDNA sequence.

[043] **Fig. 14** shows the human Kras2 isoform a cDNA sequence.

[044] **Fig. 15** shows the human Kras2 isoform b cDNA sequence.

[045] **Fig. 16** shows the mouse Kras2 isoform a cDNA sequence.

[046] Fig. 17 shows the mouse Kras2 isoform b cDNA sequence.

[047] Fig. 18 shows the mouse Ak016641 cDNA sequence.

[048] Fig. 19 shows the mouse Ak015530 cDNA sequence.

DETAILED DESCRIPTION OF THE INVENTION

5 [049] The present invention will now be described with occasional reference to the specific embodiments of the invention. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

10 [050] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to that this invention belongs. The terminology used in the description of the invention herein is for describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms “a,” “an,” and
15 “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

[051] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used in the
20 specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated, the numerical properties set forth in the following specification and claims are approximations that may vary depending on the desired properties sought to be obtained in embodiments of the present invention. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the
25 invention are approximations, the numerical values set forth in the specific examples are

reported as precisely as possible. Any numerical values, however, inherently contain certain errors necessarily resulting from error found in their respective measurements.

[052] The disclosure of all patents, patent applications (and any patents that issue thereon, as well as any corresponding published foreign patent applications), GenBank and other
5 accession numbers and associated data, and publications mentioned throughout this description are hereby incorporated by reference herein, including US Patent Application No: 20030133910 of You, et. al., filed August 23, 2002, entitled *Wild-type ras as a cancer therapeutic agent*. It is expressly not admitted, however, that any of the documents incorporated by reference herein teach or disclose the present invention.

10 [053] The present invention may be understood more readily by reference to the following detailed description of the embodiments of the invention and the Examples included herein. However, before the present methods and compositions are disclosed and described, it is to be understood that this invention is not limited to specific methods, specific nucleic acids, specific polypeptides, specific cell types, specific host cells or specific conditions, etc., as
15 such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art. It is also to be understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting.

[054] "cDNA" means a DNA prepared using messenger RNA (mRNA) as template. In
20 contrast to genomic DNA and DNA polymerized from a genomic, non- or partially-processed RNA template, cDNA contains coding sequences of the corresponding protein in the absence of introns and other non-translated nucleic acids.

[055] "Gene" refers broadly to any region or segment of DNA associated with a biological molecule or function. Thus, genes include coding sequence, and may further include
25 regulatory regions or segments required for their expression. Genes may also include non-

expressed DNA segments that, for example, from recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest, or synthesizing from known or predicted sequence information, and may include sequences encoding desired parameters.

5 [024] "Isolated," when used herein in the context of a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either dry form or an aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high
10 performance liquid chromatography. A protein that is the predominant molecular species present in a preparation is substantially purified. An isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest.

[056] "Malignant" or "cancerous" or "cancer" refers to the properties of cells or tissue that distinguish them from benign or normal cells. Malignant, cancerous, and cancer cells invade,
15 grow and destroy adjacent tissue, metastasize, and usually grow more rapidly than benign cells.

[025] "Naturally-occurring" and "wild-type," are used herein to describe something that can be found in nature as distinct from being artificially produced by man. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that
20 can be isolated from a source in nature and that has not been intentionally modified by man in the laboratory is naturally-occurring. In particular, "wild-type" is used herein to refer to the naturally-occurring or native forms of proteins and their encoding nucleic acid sequences that lack mutations or polymorphisms that alter their function. Therefore, in the context of this application, 'wild-type' includes naturally occurring variant forms of Las1 and Kras2 genes,

either representing splice variants or genetic variants between individuals, which may require different probes for selective detection.

[057] "Normal cell" means a non-cancerous or non-malignant cell.

[026] "Nucleic acid" and "polynucleotide" refer to deoxyribonucleotides or
5 ribonucleotides, nucleotides, oligonucleotides, polynucleotide polymers and fragments thereof in either single- or double-stranded form. A nucleic acid may be of natural or synthetic origin, double-stranded or single-stranded, and separate from or combined with carbohydrate, lipids, protein, other nucleic acids, or other materials, and may perform a particular activity such as transformation or form a useful composition such as a peptide
10 nucleic acid (PNA). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and may be metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions)
15 and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner et al. (1991) *Nucleic Acid Res.* 19: 5081; Ohtsuka et al. (1985) *J. Biol. Chem.* 260: 2605-2608; Cassol et al. (1992); Rossolini et al. (1994) *Mol. Cell. Probes*
20 8: 91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[058] "Proliferation" means growth and reproduction, i.e., division of cells. An important aspect of this invention is that the *Las1* and *Kras2* genes that are expressed in cells are believed to inhibit or suppress cell proliferation associated with cancer or malignancy.

25 "Inhibition" and "suppression," as used with reference to cell proliferation are terms well

known to those skilled in the art, and refer to slowing or stopping of cell division such that cells do not increase in number. The magnitude of such slowing of cell growth can be variable. Herein, any alteration of the growth of cells that comprise cancerous or precancerous cells or tissue falls within the scope of this application.

5 [027] "Sample" refers to an isolated sample of material, such as material obtained from an organism, containing nucleic acid molecules. A sample may comprise a bodily fluid; a cell; an extract from a cell, chromosome, organelle, or membrane isolated from a cell; genomic DNA, RNA, or cDNA in solution or bound to a substrate; or a biological tissue or biopsy thereof. A sample may be obtained from any bodily fluid (blood, urine, saliva, phlegm,
10 gastric juices, etc.), cultured cells, biopsies, or other tissue preparations.

[028] "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. Nucleic acids having longer sequences hybridize specifically at higher
15 temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, N.Y. Generally, highly stringent hybridization and wash conditions are selected to be 5 °C. lower than the thermal melting
20 point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize to its target subsequence, but to no other sequences. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization
25 conditions for hybridization of complementary nucleic acids that have more than 100

complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42 °C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72 °C for 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65 °C for 15 minutes (*see, Sambrook, infra.*,
5 for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45 °C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6x SSC at 40 °C for 15 minutes. For short probes (*e.g.*, 10 to 50 nucleotides), stringent conditions
10 typically involve salt concentrations of less than 1.0 M Na ion, typically 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least 30 °C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific
15 hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially similar if the polypeptides that they encode are substantially similar. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[029] "Target polynucleotide," as used herein, refers to a nucleic acid to which a
20 polynucleotide probe can hybridize by base pairing and that comprises all or a fragment of a gene that encodes Las1, Kras2 or another other Pas1 gene product. In some instances, the sequences of target and probes may be 100% complementary (no mismatches) when aligned. In other instances, there may be up to a 10% mismatch. Target polynucleotides represent a subset of all of the polynucleotides in a sample that encode the expression products of all
25 transcribed and expressed genes in the cell or tissue from which the polynucleotide sample is

prepared. The gene products of target polynucleotides are Las1 or Kras2, or other Pas1 gene products, or fragments thereof.

[030] "Target Region" means a stretch of consecutive nucleotides comprising all or a portion of a target sequence such as a gene or an oligonucleotide encoding Las1, Kras2 or another Pas1 gene product. Target regions may be 15, 16, 17, 18 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 200 or more polynucleotides in length. In some embodiments, target regions are 70 nucleotides in length, and lack secondary structure. Target regions may be identified using computer software programs such as OLIGO 4.06 software (National Biosciences, Plymouth MN), LASERGENE software (DNASTAR, Madison Wis.), MACDNASIS (Hitachi Software Engineering Co., San Francisco, Calif.) and the like.

[031] Methods For Characterizing The Etiology Of A Cancer and For Identifying An Individual Who Is At Risk Of Developing Cancer

[059] Based on the observations of Applicants, it is believed that certain cancer cells in individuals with cancer, such as adenocarcinoma of the lung, have Las1 proteins with one or more mutations, such as a single nucleotide polymorphism at codon 60. It is likewise believed that some individuals with cancer, such as adenocarcinoma of the lung, may also have reduced levels of Kras2 expression as compared to normal tissue. It is believed that individuals may be at risk for developing certain cancers, such as adenocarcinoma of the lung, if the genomic Las1 gene in such individuals' cells encode Las1 proteins with one or more mutations, such as a single nucleotide polymorphism at codon 60.

[060] Polynucleotides encoding the human and mouse Las1 protein are shown in Fig. 2 and Fig. 4; Figs. 10-19 show various of the mouse and human sequences for other genes or

cDNAs at the Pas1 locus. Polynucleotides comprising all or a portion of these sequences, or having sequences which are the complement thereof, are useful tools for designing hybridization probes for screening tissue samples for Las1 and other Pas1 gene mutations, particularly tissues from patients at risk for, known to have, or suspected of having lung cancer, and for preparing primers useful for isolating and identifying cDNA clones and genomic clones encoding the Las1 and other Pas1 genes and allelic forms thereof. Such hybridization techniques are known to those of skill in the art.

[061] In some embodiments of the invention, tissue samples are obtained from cancerous tissue or tissue that is believed to be or may become cancerous. In some embodiments, normal tissue is also obtained. According to such embodiments, a comparison may be made between the genetic profiles of the actual or suspected cancer cells and normal cells.

[062] Polynucleotide primers

[063] Primers can be used to obtain Las1 and other Pas1 polynucleotides from cDNA libraries, for screening tissue samples, or for diagnostic purposes. The primers may be used according to polymerase chain reaction (PCR) technologies to amplify transcripts of the genes which encode the Las1 and other Pas1 gene products, or portions of such transcripts. Primers may comprise 15, 16, 17, 18 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 or more nucleotides, and have a G+C content of 40% or greater. Such oligonucleotides can be at least 98%, 99% or more complementary with a portion of the DNA strand, i.e., the sense strand, which encodes the respective Las1 or other Pas1 gene or a portion of its corresponding antisense strand. Primers that have 100% complementarity with the antisense strand of a double-stranded DNA

molecule which encodes a Las1 or other Pas1 gene product have a sequence which is identical to a sequence contained within the sense strand.

[064] Isolated allele specific primers can be used for diagnosis of an individual having or at risk of developing cancer, particularly lung cancer, more particularly adenocarcinoma of the

5 lung. Allele specific primers for Las1 or other Pas1 genes are produced based upon identification of regions within the Las1 or other Pas1 gene encoding one or more polymorphisms, or SNPS, such as the polymorphism identified at Las1 codon 60. In some embodiments, the primers of the invention are designed to hybridize to the upstream and downstream (e.g., flanking) sequences of target regions of the Las1 or other Pas1 gene so as

10 to bracket the locus of such one or more SNPs.

[065] The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide specific initiation of polymerization on a significant number of nucleic acids flanking the polymorphic locus. Conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as

15 DNA polymerase, and a suitable temperature and pH. In some embodiments, primers are single stranded for maximum efficiency in amplification. Primer length is determined based on many factors, including temperature, buffer, and nucleotide composition.

[066] Primers are typically sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other

20 words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the target sequence, for example, the Las1 coding sequence, to hybridize therewith and permit amplification of one or more polymorphic locus, such as the SNP at codon 60.

[067] The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated

25 embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used

as starting materials and may be synthesized as described by Beaucage, et al. (Tetrahedron Letters, 22:1859-1862, 1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

[068] Las1-designed primers may be used in RT-PCR to quantify the amount of Las1 mRNA in the test tissues and cells. Examples of such primers include, but are not limited to:

[069] 5'- GACCAAAGCCGAGCGACTGCGGC;

[070] 3'-TCGAAGAAGTAGTTCTGTGGC

[071] Alternatively, Las1-designed primers may be used to analyze tissue sections from individuals by an RT in situ-PCR hybridization protocol as described Nuovo et al (1994) in

Am J. Pathol., 144, 659-666, which is specifically incorporated herein by reference.

[072] Polynucleotide Probes

[073] Polynucleotide probes are useful for detecting transcripts of the genes which encode the Las1 and Kras2 proteins and other Pas1 gene products. Such polynucleotide probes may comprise 15, 16, 17, 18 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, Or 200 or more nucleotides. Polynucleotide probes have a sequence which is 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more complementary with a contiguous sequence contained within the

sense strand or antisense strand of a double stranded DNA molecule which encodes the Las1 or Kras2 protein (i.e., the target region of the Las1 or Kras2 gene). Polynucleotide probes bind to the sense strand or antisense under stringent conditions, and in some instances under highly stringent conditions. The polynucleotide probes may be used in Northern assays to detect transcripts of Las1 homologous genes and in Southern assays to detect Las1 homologous genes. At least some of said polynucleotide probes comprise a polynucleotide

sequence that is complementary to a target region of a *Las1* or *Kras2* gene or one or more other *Pas1* genes.

[074] The polynucleotide probes may be genomic DNA or cDNA or mRNA, or any RNA-like or DNA-like material, such as peptide nucleic acids, branched DNAs and the like. The polynucleotide probes may be sense or antisense polynucleotide probes. Where target polynucleotides are double stranded, the probes may be either sense or antisense strands. Where the target polynucleotides are single stranded, the nucleotide probes may be complementary single strands.

[075] The polynucleotide probes may be prepared by a variety of synthetic or enzymatic schemes that are well known in the art. The polynucleotide probes can be synthesized, in whole or in part, using chemical methods well known in the art Caruthers et al. (1980) *Nucleic Acids Res. Symp. Ser.* 215-233). Alternatively, the probes can be generated, in whole or in part, enzymatically.

[076] Nucleotide analogues can be incorporated into the polynucleotide probes by methods well known in the art. The incorporated nucleotide analogues should serve to base pair with target polynucleotides. For example, certain guanine nucleotides can be substituted with hypoxanthine, which base pairs with cytosine residues. However, these base pairs are less stable than those between guanine and cytosine. Alternatively, adenine nucleotides can be substituted with 2,6-diaminopurine that can form stronger base pairs than those between adenine and thymidine. Additionally, the polynucleotide probes can include nucleotides that have been derivatized chemically or enzymatically. Typical chemical modifications include derivatization with acyl, alkyl, aryl or amino groups.

[077] The polynucleotide probes may be labeled with one or more labeling moieties to allow for detection of hybridized probe/target polynucleotide complexes. The labeling moieties can include compositions that can be detected by spectroscopic, photochemical,

biochemical, bioelectronic, immunochemical, electrical, optical or chemical means. The labeling moieties include radioisotopes, such as P^{32} , P^{33} or S^{35} , chemiluminescent compounds, labeled binding proteins, heavy metal atoms, spectroscopic markers, such as fluorescent markers and dyes, magnetic labels, linked enzymes, mass spectrometry tags, spin labels, electron transfer donors and acceptors, and the like.

[078] The polynucleotide probes can be immobilized on a substrate. Preferred substrates are any suitable rigid or semi-rigid support, including membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles and capillaries. The substrate can have a variety of surface forms, such as wells, trenches, pins, channels and pores, to which the polynucleotide probes are bound. Preferably, the substrates are optically transparent.

[079] Target Polynucleotides

[080] In order to conduct sample analysis, a sample containing polynucleotides that will be assessed for the presence of target polynucleotides, that is, *Las1* or *Kras2* genes, or *Las1* genes containing one or more SNPS, are obtained. The samples can be any sample containing target polynucleotides and obtained from any bodily fluid (blood, urine, saliva, phlegm, gastric juices, etc.), cultured cells, biopsies, or other tissue preparations. In some embodiments, samples comprise cancer cells, other cells, or cell extracts from an individual or is at risk of developing, has or may have cancer, such as adenocarcinomas of the lung.

[081] Any nucleic acid specimen, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it contains, or is suspected of containing, the specific nucleic acid sequence containing the polymorphic locus. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In

addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified, i.e., the polymorphic locus, may be a fraction
5 of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

[082] DNA utilized herein may be extracted using one of a variety of techniques such as
10 that described by Maniatis, et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y., pp 280, 281, 1982). If the extracted sample is impure, it may be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow
15 amplification to occur much more readily. Additional methods of purification of nucleic acids are described in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Elsevier, New York N.Y. In one case, total RNA is isolated using the TRIZOL reagent (Life Technologies, Gaithersburg Md.), and mRNA is isolated using oligo d(T)
20 column chromatography or glass beads. Alternatively, when polynucleotide samples are derived from an mRNA, the polynucleotides can be a cDNA reverse transcribed from an mRNA, an RNA transcribed from that cDNA, a DNA amplified from that cDNA, an RNA transcribed from the amplified DNA, and the like. When the polynucleotide is derived from DNA, the polynucleotide can be DNA amplified from DNA or RNA reverse transcribed from
25 DNA.

[083] Suitable methods for measuring the relative amounts of the target polynucleotide transcripts in samples of polynucleotides are Northern blots, RT-PCR, or real-time PCR, or RNase protection assays. For ease in measuring the transcripts for target polynucleotides, it is preferred that arrays as described above be used.

5 [084] The target polynucleotides may be labeled with one or more labeling moieties to allow for detection of hybridized probe/target polynucleotide complexes. The labeling moieties can include compositions that can be detected by spectroscopic, photochemical, biochemical, bioelectronic, immunochemical, electrical, optical or chemical means. The labeling moieties include radioisotopes, such as P^{32} , P^{33} or S^{35} , chemiluminescent compounds,
10 labeled binding proteins, heavy metal atoms, spectroscopic markers, such as fluorescent markers and dyes, magnetic labels, linked enzymes, mass spectrometry tags, spin labels, electron transfer donors and acceptors, and the like.

[085] Genetic Analysis

[086] In one type of analysis, DNA isolated from the target tissue sample is analyzed by
15 polymerase chain reaction (PCR). Regions of the Las1 ORF, or surrounding areas, are chosen and PCR primers are made that hybridize with the genomic DNA in the region. Such primers can be made to any known sequence within the Las1 gene or to regions surrounding the Las1 gene where the genomic sequence is known. One such set of regions surrounding the Las1 gene that can be used are polymorphic microsatellite markers, whose sequences and
20 locations throughout the human, and some animal genomes, are known in the art. The primers are used in a PCR reaction to amplify the region of the genome that contains the Las1 gene. A single PCR reaction may be used to amplify the entire genomic region containing the Las1 gene. Alternatively, multiple PCR reactions, each amplifying a different region of the Las1 gene may be used. Preferably, PCR reactions are used such that the entire coding

region of the Las1 gene is amplified. In addition, genomic regions within introns and surrounding the Las1 gene may also be amplified.

[087] The amplified product may be detected by analyzing via a Southern blotting technique or similarly, using dot blot analysis. Suitable solid supports useful in Southern blotting techniques are membranes, beads, microtiter plates, etc. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled. A detectable label is one that can be detected by physiochemical means, such as with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, by color absorbance, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation.

[088] Analysis of the size of a particular PCR product from the tumor or cancer cell genome as compared to the size of the same PCR product using DNA from a control cell (i.e., one known to have Las1 genes), can detect insertions or deletions of DNA in that area of the genome. It is well known in the art, that if there is an insertion of DNA in the area of a genome between the regions where two PCR primers are used to amplify the genome, the resulting PCR product is larger in size compared to the size of the same PCR product obtained using DNA from a genome where no insertion has occurred. Likewise, a deletion of DNA in the genome between two PCR primers results in a PCR product that is smaller in size compared to a control PCR product obtained using DNA from a genome not containing a deletion. Such analyses detect relatively large changes (e.g., minimum of 10% change) in size of a PCR product as compared to the product from a Las1 genome. Normally, size determination of PCR products is performed by comparing the relative sizes of two or more PCR products. For example, the size of a PCR product from a genome where a Las1

mutation is suspected is compared to the size of the same PCR product from a genome where Las1 mutations are known not to be present. Relative sizes are easily compared using migration of PCR products in an electric field, as occurs in gel electrophoresis. Agarose gel electrophoresis is often used for this purpose.

- 5 [089] Another method for analyzing PCR products is through determination of the nucleotide sequence of all or part of the PCR product. This method of analysis detects changes in relative size of PCR products that are less than 10%. This method also detects changes in the DNA sequence that do not result in relative size changes. For example, determination of the sequence and comparison of the sequence of the same PCR product
- 10 obtained from amplification of DNA from two different cells can detect single or multiple nucleotide base changes, substitutions of regions of DNA, and the like. Methods for DNA sequence determination and for DNA sequence determination of PCR products are well known in the art of molecular biology. The chain termination method of sequencing is often used. DNA sequencing is often performed by automated sequencing machines.
- 15 [090] In another type of analysis, RNA, preferably mRNA isolated from the tumor or cancer cells is used as a template to make DNA in a reverse transcription reaction. The reverse transcribed DNA is then used as a template in PCR reactions using PCR primers with sequences known to be within the mRNA of the Las1 gene. Various mRNA primers can be chosen, as described above in order to amplify the entire length of the mRNA sequence of the
- 20 Las1 gene. This can be done using a single PCR reaction, or multiple PCR reactions as described above. Analysis of the PCR products is then performed much as already described. In one type of analysis, the presence of absence of a PCR product, or a change in its size as compared to controls is indicative of large changes, such as large insertions or deletions within the Las1 genome regions. Again, such analysis is commonly performed using gel

electrophoresis of the PCR products. In another type of analysis, the DNA sequence of the PCR products is determined, using methods well known in the art.

[091] According to one embodiment, a Las1 mutating polymorphism may be detected using the reverse dot blot hybridization technique (RDB) (see for example, Bray, et al., Blood, 84(12):4361, 1994, incorporated herein by reference). Briefly, allele-specific oligonucleotides are fixed to a solid support (e.g., a filter). Typically, an amino group is added to the terminus of the allele-specific oligonucleotides for covalent attachment to the support. Labeled (e.g., biotinylated) oligonucleotides flanking the polymorphic sequence in genomic are used to amplify genomic DNA by PCR, for example, and these PCR products are denatured into single stranded DNA and hybridized to the filters containing the allele-specific oligonucleotides s.

[092] Other methods, well known in the art, can also be used to assay for presence of Las1 genes, transcripts, or changes in either as compared to wild type Las1. Some of these methods include Southern blotting, Northern blotting, RNase protection assays, S1 nuclease assays and the like.

[093] Methods of Cancer Detection by measuring protein levels

[094] In another embodiment, the invention provides for the diagnosis of an individual having or at risk of developing cancer, such as adenocarcinoma of the lung, using an antibody or other agent which detects a mutating polymorphism, such as a mutation in the Las1 protein. In some embodiments, mutations in other Pas1 genes may be detected using antibodies. Antibodies may be used either to detect levels of gene products of Las1 or one or more of the other Pas1 genes, or to detect mutant forms of the gene products.

[095] Monoclonal antibodies useful for immunophenotyping are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably

labeled in various ways. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the platelet antigens using monoclonal antibodies can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples (e.g., blood). Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

[096] The term "immunometric assay" or "sandwich immunoassay", includes simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies as described herein will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

[097] Monoclonal antibodies can be bound to many different carriers and used to detect the presence and phenotype of Las1 or other Pas1 gene products. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

[098] In performing the assays it may be desirable to include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, proteases, or anti-heterophilic immunoglobulins to anti-P1 immunoglobulins present in the experimental sample do not cross-link or destroy the

antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

[0099] It has been found that a number of nonrelevant (i.e., nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g., IgG1, IgG2a, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-100 .mu.g/II) may be important, in order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in the specimen.

[0100] Methods for Treating Individuals who have or at Risk of Developing Cancer

[0101] Various embodiments of the invention provides methods for preventing the formation of cancer or treating cancer in individuals in need of such treatment. As described herein, individuals may be identified as having cancer, such as adenocarcinoma of the lung, wherein at least one causative factor in their disease is the presence of a mutation in the Las1 gene, or in the Las1 gene and one or more of the other Pas1 genes. Other individuals may be identified as being at risk for developing a cancer, such as adenocarcinoma of the lung, wherein at least one indicator of such risk is the presence of a mutation in the Las1 gene, or in the Las1 gene and one or more of the other Pas1 genes. Such individuals are in need of treatment to prevent or stop proliferation of cancer cells. The methods of treatment described herein involve, in some embodiments, elevating the levels of Las1 protein in the individual.

[0102] In some embodiments the level Las1 protein is elevated by administering to an individual in need of treatment a Las1 protein, or a pharmaceutical composition containing a Las1 protein. In other embodiments the level Las1 protein is elevated by administering to an individual in need of treatment a polynucleotide encoding a Las1 protein, or a pharmaceutical composition containing a polynucleotide encoding a Las1 protein.

[0103] Las1 Protein

[0104] The present invention identifies a novel tumor suppressor protein, Lung Adenoma Susceptibility-1, referred to herein as Las1 or Las1 protein, the human sequence for which is shown in Fig. 1, and the mouse sequence for which is shown in Fig. 3. Polynucleotide sequences for the mouse and human Las1 open reading frames are shown in Figs. 2 and 4.

5 Alignment of amino acid sequences for Las1 in human, mouse, rat, and sea squirt are shown in Fig. 6.

[0105] The Las1 protein, and functional fragments thereof (collectively, "Las1 protein(s)"), may be produced by conventional peptide synthesizers. The Las1 proteins may also be produced using cell-free translation systems and RNA molecules derived from DNA
10 constructs that encode the Las1 proteins. Las1 proteins may also be made by transfecting host cells with expression vectors that comprise a DNA sequence that encodes the respective Las1 protein or and then inducing expression of the protein in the host cells. For recombinant production, recombinant constructs comprising a sequence which encodes the Las1 protein are introduced into host cells by conventional methods such as calcium phosphate
15 transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape lading, ballistic introduction or infection.

[0106] The Las1 protein may be expressed in suitable host cells, such as for example, mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters
20 using conventional techniques. Following transformation of the suitable host strain and growth of the host strain to an appropriate cell density, the cells are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification of the Las1 protein.

[0107] Conventional procedures for isolating recombinant proteins from transformed host
25 cells, such as isolation by initial extraction from cell pellets or from cell culture medium,

followed by salting-out, and one or more chromatography steps, including aqueous ion exchange chromatography, size exclusion chromatography steps, and high performance liquid chromatography (HPLC), and affinity chromatography may be used to isolate recombinant Las1 protein.

5 [0108] Inhibiting Lung Cancer Cell Proliferation with Las1 Protein

[0109] The present invention provides methods for inhibiting or suppressing growth of cells by introduce Las1 proteins into cells of an individual who has developed or is at risk of developing cancer. Such individuals include those who have or may develop adenocarcinomas of the lung. There are a variety of methods known in the art for introducing
10 proteins into cells. According to one method, proteins are coupled or fused to short peptides that direct entry of the Las1 protein into cells. One such group of peptides are called protein transduction domains. Another method for introduction proteins into cells uses lipid carriers. For example, proteins that are associated with liposomes are able to enter cells when the liposomes enter or fuse with the cell membranes.

15 [0110] Such methods include, but are not limited to, "protein transduction" or "protein therapy" as described in publications by Nagahara et al. (Nagahara, et al., 1998, Nat Med, 4:1449-52.) and in publications from the laboratory of Dowdy (Nagahara, et al., 1998, Nat Med, 4:1449-52.; Schwarze, et al., 1999, Science, 285:1569-72.; Vocero-Akbani, et al., 2000, Methods Enzymol, 322:508-21; Ho, et al., 2001, Cancer Res, 61:474-7.; Vocero-Akbani, et
20 al., 2001, Methods Enzymol, 332:36-49; Snyder and Dowdy, 2001, Curr Opin Mol Ther, 3:147-52.; Becker-Hapak, et al., 2001, Methods, 24:247-56.), publications which are incorporated herein by reference.

[0111] In one embodiment, an eleven amino acid sequence, the "protein transduction domain" (PTD), from the human immunodeficiency virus TAT protein (Green and
25 Loewenstein, 1988, Cell, 55:1179-88.; Frankel and Pabo, 1988, Cell, 55:1189-93.) is fused to

the Las1 protein. The purified protein is then put in contact with the surface of cells and the cells take up the Las1 protein which functions to inhibit or suppress growth of that cell. In the case where it is desired to introduce the Las1 protein containing the fused PTD into cells comprising a tumor in a human or animal, the protein is administered to the human by a variety of methods. The Las1 protein may be administered by injection (e.g., intravenously) or by inhalation in an aerosol.

[0112] Las1 proteins that contain the fused PTD are preferably made by fusing the DNA sequence encoding the Las1 gene with the DNA sequence encoding the PTD. The resulting Las1 -PTD fusion gene may be incorporated into a vector, for example a plasmid or viral vector, that facilitates introduction of the fusion gene into a organism and expression of the gene at high levels in the organism such that large amounts of the fusion protein are made therein. One such organism in which the vector containing the fusion gene can be expressed is a bacterium, preferably *Escherichia coli*. Other organisms are also commonly used by those skilled in the art. After the fusion protein is expressed at a high level in any of these organisms, the fusion protein is purified from the organism using protein purification techniques well known to those skilled in the art.

[0113] Las1 Polynucleotides

[0114] The present invention provides isolated polynucleotides which encode a Las1 protein. The Las1-encoding polynucleotides may be single-stranded or double stranded. Such polynucleotides may be DNA or RNA molecules. In one embodiment the isolated polynucleotide comprises all or a portion of the Las1 sequence shown in Fig. 2 or Fig. 4. The Las1 polynucleotides are useful in one embodiment for preparing Las1 proteins.

[0115] The present invention also encompasses isolated polynucleotides whose sequence is the complement of the Las1 gene sequence, shown in Figs. 2 and 3, and polynucleotides that

hybridize under stringent conditions, in some embodiments under highly stringent conditions, to the open reading frame sequence of the Las1 gene sequence, or the complement thereof.

[0116] Polynucleotides comprising sequences encoding a Las1 protein may be synthesized in whole or in part using chemical methods. Polynucleotides which encode a Las1 protein, particularly alleles of the genes which encode a Las1 protein, may be obtained by screening a genomic library or cDNA library with a probe comprising sequences identical or complementary to the sequences shown in Figs. 2 or 3, or with antibodies immunospecific for a Las1 protein, to identify clones containing such polynucleotide. Alternatively, polynucleotides encoding Las1 proteins may be made using polymerase chain reaction (PCR) technology and primers that bind specifically to sequences which are known to encode a Las1 protein.

[0117] Inhibiting Lung Cancer Cell Proliferation with Las1 polynucleotides

[0118] In one aspect, the present method comprises introduction of Las1 encoding polynucleotides, preferably contained within a vector, into cancer cells so that the cells achieve increased levels of Las1 expression. Herein, such introduction or transfer of a DNA molecule or molecules, specifically a DNA molecule encoding one or more Las1 encoding polynucleotide, into a cell refers to any of a variety of methods known in the art to achieve transfer of DNA molecules into cells. Whatever methodology is used to administer the Las1 genes to humans or animal, such methodologies comprise variations that result in the Las1 genes being introduced exclusively into normal and not being introduced into tumor cells. For example, techniques are known in the art that result in recombinant viruses specifically infecting certain cell types within a human or animal. For viruses, such "targeting" can be accomplished through manipulation of cellular receptors for the recombinant viruses and/or manipulation of viral ligands that recognize and bind to cellular receptors for the viruses.

Such methodologies, as used to introduce Las1 genes into cancer cells in animals or humans,

are within the purview of the present application. Targets on cancer cells include, but are not limited to, proteins such as carcino embryonic antigen, and other markers that are differentially expressed on cancer cells but not in corresponding normal cells. Specific ligands for such targets include, but are not limited to, known ligands, antibodies.

- 5 [0119] In one embodiment, polynucleotides encoding the Las1 protein or a functional equivalent thereof are introduced into such cells to permit expression or overexpression of the Las1 protein. Viral or plasmid vectors may be used to deliver the polynucleotide to the cells. Levels of Las1 may be increased in cancer cells by introducing a DNA fragment comprising an Las1 polynucleotide and a promoter into the cell and expressing the Las1 protein.
- 10 Preferably, the promoter, which is operably linked to the Las1 polynucleotide is a tissue specific promoter. The DNA fragment may be incorporated into a viral vector or into a liposome which, preferably, further comprises a molecule which targets the liposome to the cancer cell.

- [0120] In one embodiment, polynucleotides encoding the Las1 protein or a functional
- 15 equivalent or fragment thereof is introduced into cancer cells to permit expression or overexpression of the Las1 protein. In one embodiment, Las1 delivery is specifically selective for cancer cells and is achieved using a targeting carrier that is selective for cancer cells and does not direct delivery to normal cells.

- [0121] In order to introduce the polynucleotide sequences encoding Las1 activity into cells,
- 20 the protein coding region of the polynucleotide sequences is normally attached to sequences that facilitate its transcription into mRNA as well as translation of the mRNA into Las1. A strategy common in the art for doing this is to clone the polynucleotide sequence encoding the Las1 protein into a vector which contains sequences facilitating expression of a protein coding sequence cloned therein.

[0122] Expression vectors normally contain sequences that facilitate gene expression. An expression vehicle can comprise a transcriptional unit comprising an assembly of a protein encoding sequence and elements that regulate transcription and translation. Transcriptional regulatory elements generally include those elements that initiate transcription. Types of such elements include promoters and enhancers. Promoters may be constitutive, inducible or tissue specific. Transcriptional regulatory elements also include those that terminate transcription or provide the signal for processing of the 3' end of an RNA (signals for polyadenylation). Translational regulatory sequences are normally part of the protein encoding sequences and include translational start codons and translational termination codons. There may be additional sequences that are part of the protein encoding region, such as those sequences that direct a protein to the cellular membrane, a signal sequence for example.

[0123] The Las1-encoding polynucleotides that are introduced into cells are, in some embodiments, expressed at a high level (i.e., the introduced polynucleotide sequence produces a high quantity of Las1 protein within the cells) after introduction into the cells. Techniques for causing a high-level of expression of polynucleotide sequences introduced into cells are well known in the art. Such techniques frequently involve, but are not limited to, increasing the transcription of the polynucleotide sequence, once it has been introduced into cells. Such techniques frequently involve the use of transcriptional promoters that cause transcription of the introduced polynucleotide sequences to be initiated at a high rate. A variety of such promoters exist and are well known in the art. Frequently, such promoters are derived from viruses. Such promoters can result in efficient transcription of polynucleotide sequences in a variety of cell types. Such promoters can be constitutive (e.g., CMV enhancer/promoter from human cytomegalovirus) or inducible (e.g., MMTV enhancer/promoter from mouse mammary tumor virus). A variety of constitutive and

inducible promoters and enhancers are known in the art. Other promoters that result in transcription of polynucleotide sequences in specific cell types, so-called "tissue-specific promoters," can also be used. A variety of promoters that are expressed in specific tissues exist and are known in the art. For example, promoters whose expression is specific to neural, liver, epithelial and other cells exist and are well known in the art. Methods for making such DNA molecules (i.e., recombinant DNA methods) are well known to those skilled in the art.

[0124] Vectors for introducing Las1 polynucleotides into target cells

[0125] In the art, vectors refer to nucleic acid molecules capable of mediating introduction of another nucleic acid or polynucleotide sequence to which it has been linked into a cell. One type of preferred vector is an episome, i.e., a nucleic acid capable of extrachromosomal replication. Other types of vectors become part of the genome of the cell into which they are introduced. Vectors capable of directing the expression of inserted DNA sequences are referred to as "expression vectors" and may include plasmids, viruses, or other types of molecules known in the art.

[0126] Typically, vectors contain one or more restriction endonuclease recognition sites which permit insertion of the Las1 polynucleotide sequence. The vector may further comprise a marker gene, such as for example, a dominant antibiotic resistance gene, which encode compounds that serve to identify and separate transformed cells from non-transformed cells.

[0127] One type of vector used in the present invention is selected from viral vectors. Viral vectors are recombinant viruses which are generally based on various viral families comprising poxviruses, herpesviruses, adenoviruses, parvoviruses and retroviruses. Such recombinant viruses generally comprise an exogenous polynucleotide sequence (herein, a

Las1 gene) under control of a promoter which is able to cause expression of the exogenous polynucleotide sequence in vector-infected host cells.

[0128] One type of viral vector is a defective adenovirus which has the exogenous polynucleotide sequence inserted into its genome. The term "defective adenovirus" refers to

5 an adenovirus incapable of autonomously replicating in the target cell. Generally, the genome of the defective adenovirus lacks the sequences necessary for the replication of the virus in the infected cell. Such sequences are partially or, preferably, completely removed from the genome. To be able to infect target cells, the defective virus contains sufficient sequences from the original genome to permit encapsulation of the viral particles during *in*
10 *vitro* preparation of the construct. Other sequences that the virus contains are any such sequences that are said to be genetically required "in *cis*."

[0129] It is desirable that the adenovirus is of a serotype which is not pathogenic for man. Such serotypes include type 2 and 5 adenoviruses (Ad 2 or Ad 5). In the case of the Ad 5 adenoviruses, the sequences necessary for the replication are the E1A and E1B regions.

15 Methods for preparing adenovirus vectors are described in U.S. Patent No. 5,932,210, which issued in August, 1999 to Gregory et al., U.S. Patent No. 5,985,846 which issued in November, 1999 to Kochanek et al, and U.S. Patent No. 6,033,908 which issued in March, 2000, to Bout et al.

[0130] It is also desirable that the virus vector is an immunologically inert adenovirus. As
20 used herein the term "immunologically inert" means the viral vector does not encode viral proteins that activate cellular and humoral host immune responses. Methods for preparing immunologically inert adenoviruses are described in Parks et al., *Proc Natl Acad Sci USA* 1996; 93(24) 13565-70; Leiber, A. et al., *J. Virol.* 1996; 70(12) 8944-60; Hardy s., et al, *J. Virol.* 1997, 71(3): 1842-9; and Morsy et al, *Proc. Natl. Acad. Sci. USA* 1998. 95: 7866-71,

25 all of which are specifically incorporated herein by reference. Such methods involve Cre-

loxP recombination. In vitro, Cre-*loxP* recombination is particularly adaptable to preparation of recombinant adenovirus and offers a method for removing unwanted viral nucleotide sequences. Replication deficient recombinant adenovirus lacks the E1 coding sequences necessary for viral replication. This function is provided by 293 cells, a human embryonic kidney cell line transformed by adenovirus type. First generation adenoviruses are generated by co-transfecting 293 cells with a helper virus and a shuttle plasmid containing the foreign gene of interest. This results in the packaging of virus that replicates both the foreign gene and numerous viral proteins. More recently, 293 cells expressing Cre recombinase, and helper virus containing essential viral sequences and with a packaging signal flanked by *loxP* sites, have been developed (See Parks et al.) In this system, the helper virus supplies all of the necessary signals for replication and packaging *in trans*, but is not packaged due to excision of essential sequences flanked by *loxP*. When 293-Cre cells are co-transfected with this helper virus, and a shuttle plasmid (pRP1001) containing the packaging signal, nonsense "filler DNA", and the foreign gene, only an adenovirus containing filler DNA and the foreign gene is packaged (LoxAv). This results in a viral recombinant that retains the ability to infect target cells and synthesize the foreign gene, but does not produce viral proteins.

[0131] Another type of viral vector is a defective retrovirus which has the exogenous polynucleotide sequence inserted into its genome. Such recombinant retroviruses are well known in the art. Recombinant retroviruses for use in the present invention are preferably free of contaminating helper virus. Helper viruses are viruses that are not replication defective and sometimes arise during the packaging of the recombinant retrovirus.

[0132] Non-defective or replication competent viral vectors can also be used. Such vectors retain sequences necessary for replication of the virus. Other types of vectors are plasmid vectors.

[0133] After Las1-encoding polynucleotides are introduced into cells, techniques may be used to determine the cells into which the polynucleotide sequences have been introduced and/or the specific cells that are expressing the introduced polynucleotide sequences. A variety of techniques to examine the presence of polynucleotide sequences and/or expression of polynucleotide sequences exist and are well known in the art. Some such techniques include Southern blotting, Northern blotting, polymerase chain reaction (PCR), Western blotting, RNase protection, radioiodide uptake assays, and others.

[0134] Also encompassed by the present invention, are single stranded polynucleotides, hereinafter referred to as antisense polynucleotides, having sequences which are complementary to the DNA and RNA sequences which encode the Las1 protein. The term complementary as used herein refers to the natural binding of the polynucleotides under permissive salt and temperature conditions by base pairing.

[0135] Administration of proteins and polynucleotides

[0136] Doses may be selected, depending on their dosage form, patient's age, sex and severity of disease, and other conditions, as appropriate, but the amount of the active ingredient may be generally about 0.0001 to 100 mg/kg a day. A unit dosage form may contain about 0.001 to 1000 mg of the active ingredient. The compositions may be administered using any mode that is medically acceptable, meaning any mode that produces effective levels of the active protein without causing clinically unacceptable adverse effects.

Such modes of administration include parenteral routes (e.g., intravenous, intra-arterial, subcutaneous, intramuscular, mucosal or infusion), but may also include oral, rectal, topical, nasal or intradermal routes. Another route of introduction, of special use for treatment of patients with pulmonary fibrosis, is the respiratory route by inhalation into the lungs. Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations, increasing convenience to the

patient and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. The pharmaceutical compositions of the present invention may also be administered by the respiratory route. The formulations administered by the respiratory route are generally oral aerosol formulations. Such formulations can be administered via the respiratory route in a variety of ways.

[0137] In the event that a response in an individual is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of peptides. The duration of therapy with the pharmaceutical compositions used in the methods of the present invention will vary, depending on the unique characteristics of the pharmaceutical composition and the particular therapeutic effect to be achieved, the severity of the disease being treated and the condition and potential idiosyncratic response of each patient. Ultimately the attending physician will decide on the appropriate duration of therapy with the pharmaceutical composition used in the method of the present invention.

[0138] Pharmaceutical compositions

[0139] Therapeutic proteins and polynucleotides may be administered to an individual in need of the same in a pharmaceutical composition. Suitable formulations for delivery are found in Remington's Pharmaceutical Sciences, 17th ed. (Mack Publishing Co., Philadelphia, Pa., 1985). These pharmaceutical compositions are suitable for use in a variety of drug delivery systems (Langer, Science 249:1527-1533, 1990).

[0140] Las1 and Kras2 proteins and polynucleotides in pharmaceutical compositions are suitable for single administration or in a series of inoculations. The pharmaceutical compositions are intended for parenteral, topical or oral administration. Parenteral administration may be by intravenous, subcutaneous, intradermal, intraperitoneal or

intramuscular administration. Parenteral administration may be preferentially directed to the patient's liver such as by catheterization to hepatic arteries or into a bile duct. For parenteral administration, the compositions can include Las1 proteins and a suitable sterile carrier such as water, aqueous buffer, 0.4% saline solution, 0.3% glycine, hyaluronic acid or emulsions of nontoxic nonionic surfactants as is well known in the art. The compositions may further include substances to approximate physiological conditions such as buffering agents and wetting agents such as NaCl, KCl, CaCl₂ sodium acetate and sodium lactate.

[0141] Solid compositions in conventional nontoxic solid carriers such as, for example, glucose, sucrose mannitol, sorbitol, lactose, starch, magnesium stearate, cellulose or cellulose derivatives, sodium carbonate and magnesium carbonate. For oral administration of solid compositions, the HCV-like particles preferably comprise 10% to 95%, and more preferably 25% to 75% of the composition.

[0142] Therapeutic compositions may be administered as a single dose, but more likely as a series of dosages over a period of days, weeks or even months. Herein, an effective therapeutic dose is a dose that inhibits growth of a tumor, or causes tumor regression.

EXAMPLES

[0143] Mouse model for Lung Cancer: Inbred mice models offer an effective means of identifying candidate lung cancer susceptibility loci. Inbred strains of mice have different susceptibilities to spontaneous and carcinogen-induced lung tumor formation. The A/J strain is the most susceptible to lung tumorigenesis whereas the C3H and C57BL/6 are among the most resistant strains. Linkage study using (A/J \times C3H/HeJ) F2 and (A/J \times C57BL/6J) F2 mice has demonstrated that Pas1 is the major lung tumor susceptibility locus in mice that has been mapped to the distal region of chromosome 6, and accounts for approximately 50% of the phenotypic variance. Here, Applicants provide the most definitive evidence yet to support the candidacy of both Las1 and Kras2 as the Pas1 genes on mouse chromosome 6.

This work represents the identification of candidates for the first major lung cancer QTL in either mouse models or humans since the initial mapping of Pas1 QTL in 1993.

[0144] Materials and Methods for Construction of Congenic Strains

Inbred A/J and C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The basic

5 breeding scheme in the study was to put an approximately 26.1 cM fragment of chromosome 6, encompassed by D6MIT54 and D6MIT373 markers from lung tumor susceptible A/J strain onto the genetic background of lung tumor resistant C57BL/6J mice. A/J mice were initially crossed to C57BL/6J mice. F1 progeny were backcrossed to C57BL/6J mice to produce the first backcross generation (N2). The N2 generation heterozygous for the chromosome region

10 of interest was then backcrossed again to C57BL/6J mice to produce the N3 generation. This process was repeated for a total of seven backcrosses. At N5 generation, additional microsatellite markers on chromosomes 9, 10, 17, and 19, including D9MIT75, D9MIT355, D9MIT35, D10MIT106, D10MIT2, D10MIT126, D17MIT246, D17MIT23, D17MIT50, D19MIT36, D19MIT10, and D19MIT89, were screened to obtain the optimal breeders which
15 harbor the least amount of the non-Pas1 donor genome. At N8, 132 male substrains containing different chromosomal regions of interest were generated. These individual substrains were then each crossed to 3 C57BL/6J females to produce the N9 generation. After selection, an average of 5-12 N9 congenic mice were generated from each subcongenic strain.

20 [0145] Genotyping Using Polymorphic Markers

For selecting mice on the basis of their geneotypes throughout the Pas1 region on chromosome 6, the following markers were used: D6MIT54, D6MIT52, D6MIT59, D6MIT57, D6MCO10, D6MCO11, D6MIT15, and D6MIT373. All of the mouse microsatellite primers were purchased from Research Genetics, Inc. (Huntsville, AL). The forward primer was end-labeled with 32P-ATP, and 30 cycles of
25 PCR were performed at 94°C for denaturation, 55°C for annealing and 72°C for extension.

Eight percent denaturing polyacrylamide gels were used for resolution of the radiolabeled PCR products followed by autoradiography.

[0146] Lung Tumorigenesis in Congenic Mice

Five-week-old N9 mice were given a single i.p. injection of urethane (1 mg/g body weight) in 0.2 ml PBS. All animals were euthanized by CO₂ asphyxiation 7.5 months after urethane initiation. A portion of lung tumors and normal tissue were removed and flash frozen in liquid nitrogen. The remaining was fixed in Tellyesniczky's solution and examined with the aid of a dissecting microscope to count and size the tumors. Tumor volumes were determined by measuring the three-dimensional size of each tumor and by using the average of the three measurements as the diameter. The radius (diameter/2) was determined, and the total tumor volume was calculated by: Volume = $\frac{4}{3}\pi r^3$ (r-radius). Two-way ANOVA was used to determine the difference in both the number and the size of lung tumors between control and congenic groups.

[0147] Northern Blot Analysis and Semiquantative RT-PCR.

Total RNAs were prepared from mouse lung tissues using TRIzol reagent (Life Technologies). Poly(A)⁺ RNAs were purified from the total RNAs with MicroPoly(A)Pure (Ambion). A 2-microgram aliquot of each Poly(A)⁺ RNA was separated on a 1% agarose gel containing 2% formaldehyde and transferred to nylon membrane. The blots were hybridized with a random-primed ³²P-labelled cDNA probe in ExpressHybTM Hybridization Solution (Clontech) at 68 °C, washed with 0.1XSSC-0.1%SDS at 50-65 °C, and exposed for autoradiography at -80 °C. For semiquantative RT-PCR, first strand cDNAs were synthesized using SuperScript 2 (Life Technologies) with random primer and 1 mg of Poly(A)⁺ RNAs or 3 µg of total RNAs described above. Primer sequences were 5'- GACCAAAGCCGAGCGACTGCGGC, 3'- TCGAAGAAGTAGTTCTGTGGC for Las- 1, 5'-TGACATCCGTAAAGACCTCTATGCC, 3, -AAG CAC TTG CGG TGCACG ATG GAG for b-actin. All reactions involved initial denaturation at 94 °C for 3 min followed by 30-35 cycles at 94 °C for 30 sec, 55 °C for 30sec,

72 °C for 30 sec (for Las1), and 21 cycles at 94 °C for 30 sec, 68 °C for 30sec, 72 °C for 30 sec (for α -actin) on PTC- 100 Programmable Thermal Controller (MJ Research).

[0148] Colony Formation Assay and Athymic Mouse Tumorigenicity Assay. To obtain the

entire sequence of cDNAs, Applicants performed Rapid Amplification of cDNA Ends

(RACE) using Marathon™ cDNA Amplification Kit (Clontech). The entire Las1 cDNAs

for both A/J and C57BL/6L were cloned into the pcDNA3.1(+) vector (Invitrogen) with the

restriction sites of HindIII and EcoRI. Various mouse lung tumor cells were used in this

experiment. LM1 is a metastatic cell line derived from A/J. MC4 and MC14 cells were from

chemical-induced lung tumors in B6C3F1. These lines and NIH3T3 (mouse fibroblast cell

line) were obtained from either American Type Cell Culture or University of Colorado. For

colony formation assay, LM1 cells were seeded at 1.5×10^6 per 10 cm dish and transiently

transfected with 4 μ g of the constructed and empty vectors with Lipofectamine (Invitrogen).

The transfected cells were cultured in the presence of 1mg/ml G418 for 2 weeks. The cells,

which survived, were fixed with 10% formalin and stained with 0.125% crystal violet. The

colonies (≥ 1 mm) were counted. Applicants repeated at least 3 independent experiments. For

athymic mouse tumorigenicity, female athymic BALB/c nude mice aged 4-6 weeks were

purchased from Charles Rivers Laboratories. Applicants injected 10 million cells

subcutaneously into each flank of nude mice. Four animals were used per sample.

Applicants monitored the health of animals 3 times a week and measured the size of tumors

weekly for 6 weeks. Tumor volume was calculated as $\text{length} \times \text{height} \times \text{width} \times 0.5$.

Applicants also confirmed the expression of Las1 for several tumors resected from nude mice

by RT-PCR.

[0149] Immunocytochemistry. pcDNA3.1(+)/N-myc-tagged Las1 expression vectors were

constructed from A/J allele and C57BL/6J allele individually to identify the localization of

Las1 in cells. Transiently transfected NIH/3T3 cells with pcDNA3.1(+)/N-myc-Las- 1 were

re-plated on multiwell chamber slides (Beckton Dickinson). Then the cells were fixed with 4% paraformaldehyde in PBS and made permeable with 0.1% Triton X-100 in PBS for 3 minutes. Cells were covered with 3% BSA-containing blocking solution for 1 hr at room temperature. Then the cells were incubated with mouse anti-myc antibody (Oncogene, 5 diluted 1:50 in blocking solution) for 1 hr at room temperature. Anti-myc antibody was stained with goat anti-mouse secondary antibody conjugated to rhodamine (1:250) for 1 hr and viewed with ECLIPSE E600 microscope (Nikon).

[0150] Sequence and Expression Analyses of Candidate Pas1 Genes. There are 12 putative

genes detected by Exon Prediction software within ~0.5 Mb Pas1 region (Table 1, shown in 10 Fig. 9). Among them, six genes, including Lrmp, Bcat1, Las1 (145.25Mb), AK015530, Kras2, and AK016641, were confirmed to be expressed in lung tissues by RT-PCR and Northern blot analyses (data not shown). Consistent with Applicants' previous study, Northern blot analysis showed that Kras2 4B and AK015530 expression in A/J mouse lung was 36% and 58% higher than that from C57BL/6J strain, respectively (Fig. 5B).

15 AK016641 contained an elevated frequency of an alternative-splicing transcript in A/J (Fig. 5B). No differential expression was found for Bcat1, Lrmp, and Las1 between lung tissues of A/J and C57BL/6J strains (Fig. 5B & Fig. 7C). Next, sequence analyses were performed to detect functional polymorphisms between susceptible and resistant strains. For Lrmp, Bcat1, Kras2, AK016641, and AK015530, the entire open reading frames (ORFs) were sequenced.

20 For Las1, additional 5' and 3' Rapid amplification cDNA ends (RACE) methods were used to obtain the entire ORF. The mouse Las1 sequence and its comparison with those of rat and human are shown in Fig. 6. As shown in Fig. 5C, four transcripts including AK016641, AK015530, Lrmp, and Las1 showed functional polymorphisms between A/J and C67BL/6J strains. AK016641 had 2 functional polymorphisms at codon 218 (Arg to His), codon 258 25 (Gly to Glu) and an alternative splicing transcript without exon 5 only found in A/J strain.

AK015530 had a polymorphism at codon 28 resulting in a change of Asp to Gly. Lrmp had 5 functional polymorphisms including codon 31 (Asp to Gly), codon 56 (Gly to Asp), codon 58 (Phe to Leu), codon 438 (Arg to Gly), and codon 537 (Pro to Leu). Las1 showed one functional polymorphism at codon 60 (Asn to Ser). Therefore, five genes including
5 AK016641, AK015530, Lrmp, Las1, and Kras2 contained either functional polymorphisms or differential expression between A/J and C57BL/6J and were further characterized as candidates for Pas1 gene(s).

[0151] Distribution Pattern and Cellular Localization of Las1. Las1 has a 2193 bp ORF and

consists of 730 amino acids; estimated molecular weight is 84.8 Kd homologous to axomonal
10 p83.9 (*Ciona intestinalis*) (Fig. 6). Northern blot showed that Las1 mRNA had a size of approximately 4 Kb and the same expression level between A/J and C57BL/6J (Fig. 7C-a).

RT-PCR data also revealed that no differential expression was detected in a total of 12 different strains examined including 6 resistant (C57BL/6J, DBA/2J, SJL/J, C3H/HeJ, AKR/J, *M. spretus*), 4 intermediate (BALB/c, 129/J, CBA/J, SM/J) and 2 susceptible (A/J,

15 SWR/J) strains (data not shown). In multiple organ expression panels, Las1 expressed at higher levels in lung, kidney, and testis in both A/J and C57BL/6J strains (Fig. 7C-b).

Moreover, in 16 mouse cell lines, LM2, Spon5, CL-13H, CL20 showed relatively high levels of Las1 expression while some lines such as LM1, CMT64, PCC4 showed extremely low expression (Fig. 7C-c). There appears a close correlation between functional polymorphism

20 of Las1 and lung tumor susceptibility in various mouse strains. Applicants sequenced the entire open reading frame of Las1 gene in 12 mouse strains including 6 resistant, 4 intermediate, and 2 susceptible strains. The 12 strains of mice fall into two genotypes according to their sequence alteration in 60th codon of Las1 gene. The susceptible/intermediate groups including 6 strains (A/J, SWR/J, BALB/c, 129/J, CBA/J,

25 SM/J) have an AAT at codon 60 encoding an asparagine, while the six resistant strains

including C57BL/6L, DBA/2J, SJL/J, C3H/HeJ, AKR/J, and *M. spretus* have an AGT at codon 60 encoding a serine (data not shown). To assess the possible function of the Las1 gene, the cellular localization of the Las1 product in NIH3T3, COS7, and LM1 cells were investigated by transient transfection N-terminus myc-tagged Las1 vectors derived from A/J and C57BL/6J alleles into NIH/3T3 cells. Fig. 7D shows the cytoplasmic distribution of the Las1 gene product in NIH/3T3 cells. Similar results were obtained when Applicants transfected those plasmids into COS7 and LM1 cells (data not shown). Looking into homology between mouse Las1 and other species, Las1 products revealed a high degree of conservation from *Ciona intestinalis* to human (Fig. 6). Las1 protein is 67% identical and 81% positive to the derived human Las1 protein (similar to human hypothetical protein FLJ10921, 30.41Mb). Searching NCBI protein database using mouse protein sequence revealed a rat homologous protein, encoded by NCBI predicted gene LOC297720 (84% identities and 92% positives). The mouse Las1 protein is also homologous to a *Ciona intestinalis* protein axonemal p83.9 (GI: 20086393, 33% identities and 52% positives) (Fig. 6). Amoxonemes are highly organized microtubule based structure present in diverse types of cells that perform motile, sensory, and developmental functions in organisms from protists to humans. These functions are consistent with observed cellular distribution.

[0152] Effect of Las1 on the Growth of Mouse Lung Tumor Cells. To further evaluate the

effects of Las1 candidates on cell proliferation, Applicants carried out a series of transfection experiments to determine whether these candidates could promote or inhibit growth of the mouse lung tumor cell lines including LM1, MC7 and MC14 cells. Among the five genes (AK016641, AK015530, Lrmp, Las1, and Kras2) tested, only Las1 showed differential effect on cell growth between the A/J allele and the C57BL/6J allele (data not shown for genes with negative results). As shown in Fig. 7A-a, transfection of Las1-A/J-pcDNA3.1 into LM1 cells produced ~90% colonies vs pcDNA3.1 control vector, while transfection of Las1-

C57BL/6J-pcDNA3.1 produced ~30% colonies vs pcDNA3.1 control vector, indicating that Las1-C57BL/6J-pcDNA3.1 significantly inhibited anchorage-dependent cell growth ($p < 0.0001$; Fig. 7A-a). Similar inhibitory effects were also observed in MC7 and MC14 cells (MC14, Fig. 7A-b; MC7, data not shown). Clearly, Las1 derived from C57BL/6J allele can suppress tumor cell growth *in vitro*. Applicants also tested the ability of the C57BL/6J derived Las1 to inhibit tumor development of LM1 cells in nude mice. As shown in Fig. 7B, after 5 weeks of inoculation, large tumors developed in all four mice injected with Las1-A/J-pcDNA3.1 transfected cells, but only small tumors developed in the four mice injected with transformed LM1 cells carrying Las1-C57BL/6J-pcDNA3.1 ($P < 0.05$) (Fig. 7B-b, c, d). Expression of Las1 was also confirmed in the resected tumors by RTPCR (Fig. 7B-a). These results indicate that the C57BL/6J derived Las1 can inhibit the tumorigenic potential of the LM1 cells *in vivo*.

[0153] Lung Tumorigenesis on F1 Kras2 Heterozygous Deficient Mice. Six-week-old male 129/Sv-K-ras+/- (Kras2 "knockout"), A/J female, and C57BL/6J female mice were paired to develop breeding colonies for production of (A/J \times 129/Sv-K-ras+/-) F1 and (C57BL/6J \times 129/Sv-K-ras+/-) F1 mice. Each F1 mouse was genotyped for the presence of the Kras2 targeted mutation and was randomized into groups according to the Kras2 genotypes and carcinogen treatments. For groups treated with urethane, animals were given a single i.p. injection of urethane (1 mg/g body weight) in 0.2 ml phosphatebuffered saline. For MNU treatment groups, all animals were given a single i.p. injection of MNU (50 mg/kg body weight) in 0.2 ml phosphate-buffered saline (PBS). Eighteen to twenty weeks after treatment with carcinogens, animals from all eight groups were euthanized by CO2 asphyxiation. For each mouse, portions of the tumors plus some normal lung were frozen in liquid nitrogen. The remaining tissue and tumors were fixed in Tellyesniczky's solution overnight, followed by 70% ethanol treatment. The tumor number and size were measured.

[0154] Kras2 Activity Assays. Mouse lung tumors from Kras2^{+/-} of A/J or C57BL/6J mice were obtained from the lung tumor bioassays. These tumors were homogenized in lysis buffer- PBS (5 mM MgCl₂, 1 mM DTT, 0.2 mM PMSF, 2µg/mL leupeptin, 5 µg/mL aprotinin, 1 mM benzamidine) and cleared by centrifugation. NP-40 was then added to 1% after centrifugation. Protein concentrations were determined by Bradford assay (BioRad) and equalized prior to incubation with 40 mg GST-RafRBD (Ras binding domain), precoupled to glutathione agarose. Half as much lysate was used in the pulldown from C57BL/6J tumors. After 2 h of mixing, beads were washed and bound proteins were eluted with SDS sample treatment buffer. Bound Kras2 protein was detected by western blot with a-KRas2 F234 antibody (Santa Cruz Biotechnology).

[0155] Statistical Analysis. To compare the tumor multiplicity and load genotype/genotype (Kras2^{+/-}/Kras2^{+/+}) ratios between (A/J¥129/Sv-K-ras^{+/-}) F1 and (C57BL/6J¥129/Sv-Kras^{+/-}) F1 strains, tumor numbers and loads were log-transformed and t-tests applied using the genotype-genotype differences of the log-transformed values. To facilitate logtransformation, zero multiplicity values were assigned a value of 0.5 and zero load values were assigned a value of 0.001. This would produce conservative p-values.

[0156] Kras2 Alleles Contribute to Differential Lung Tumor Progression. Although no functional polymorphisms were detected in the coding sequence of the Kras2 gene between A/J mice and C57BL/6J mice, Applicants further characterized its candidacy for Pas1 because of previously observed allele-specific expression and mutation of Kras2 in mouse lung tumors from hybrid mice such as A/J _ C67BL/6J F1 or A/J _ C3H/HeJ F1 in which as much as 20-fold higher expression of Kras2 mRNA from the A/J allele was observed (20). A mouse lung tumor bioassay was conducted using heterozygous Kras2 deficient mice (K-ras^{+/-}). The animals were paired to develop breeding colonies for production of (A/J¥K-ras^{+/-}) F1, and (C57BL/6J¥K-ras^{+/-}) F1 mice. Either wild type or heterozygous Kras2

knockout and wild type mice were subjected to lung tumorigenesis assays using two lung carcinogens: urethane and methylnitrosourea (MNU). Treatment of both (A/J~~Y~~K-ras+/-) F1 and (C57BL/6J~~Y~~K-ras+/-) F1 heterozygous deficient mice with urethane or MNU produced 4-5 times more tumors/mouse than did treatment of wild type mice, indicating no allelic difference was found in promoting lung tumor multiplicity as result of Kras2 heterozygous deficiency (Fig. 8A). In contrast, the allelic effects on tumor load (or tumor size) showed significant differences: a nearly 50-fold increase in tumor volume was observed in (A/J~~Y~~K-ras+/-) F1 heterozygous deficient mice when compared to wild type controls while only ~8-fold increase in tumor size was observed in (C57BL/6J~~Y~~K-ras+/-) F1 heterozygous deficient mice when compared to wild type control mice (Fig. 8B). These results indicate that the remaining A/J or C57BL/6J Kras2 allele in K-ras+/- mice has a significant differential effect on lung tumor progression (Fig. 8B; $p < 0.001$). Thus, the Kras2 allele derived from A/J mouse strains confers a significantly higher susceptibility to lung tumor progression than does the C57BL/6J Kras2 allele.

[0157] The Activation State of Kras2 in Lung Tumors from Kras2 Deficient Mice. The mechanism for the Kras2 alleles in lung tumor progression appears to be related to the observed differential mRNA expression of the activated Kras2 alleles in lung tumors from the A/J strain of mice. Interestingly, there was considerably higher active Kras2 protein expressed in lung tumors from mice containing only A/J Kras2 allele compared to mice with only C57BL/6J Kras2 allele as determined by an assay which utilized the Ras binding domain (RBD) of c-Raf to specifically recognize GTP-bound Kras2 (26; Fig. 8C). In fact, Applicants were unable to detect any active Kras2 protein in lung tumors from (C57BL/6J~~Y~~K-ras+/-) F1 mice (Fig. 8C). This result was seen despite the use of half the amount of lysate in lung tumors from (A/J~~Y~~K-ras+/-) F1 mice. Thus, mice containing only

the C57BL/6J Kras2 allele expressed less activated Kras2 protein in a fashion that associates closely with its resistance to chemically induced lung tumorigenesis.